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Molecular cloning, expression, sequence analysis and *in silico* comparative mapping of trehalose 6-phosphate gene from Egyptian durum wheat

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Trehalose is a non-reducing disaccharide which consists of two glucose units that functions as a compatible solute to stabilize the membrane structures under heat and desiccation stress. Trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) are the key enzymes for trehalose biosynthesis in the plant kingdom. On the basis of bioinformatics prediction, fragment containing an open reading frame of 945 bp was cloned from durum wheat. Sequence comparison and analysis of conserved domains revealed the presence of a *TPP* domain. Full length of the gene was isolated using gene race technology. Semi-quantitative RT-PCR and real time quantitative PCR indicated that the expression of this gene is up-regulated in response to drought stress. The biochemical assay of the trehalase activity showed that the enzyme's activity decreased under the dehydration stress. The obtained phylogenetic tree showed that the isolated TPP protein forms a distinct clad close to the *Oryza sativa* trehalose-6-phosphate phosphatase. *In silico* and comparative mapping indicated that the isolated *TPP* gene is localized on rice chromosome 8, durum wheat chromosome 20, bread wheat chromosome 3B, oat linkage group E, sorghum chromosome 4 and barley 5H.

Key words: Abiotic stress tolerance, trehalose-6-phosphate phosphatase (TPP), durum wheat, trehalose, real time PCR, cloning, full length gene, drought stress.

INTRODUCTION

The global food situation is currently being redefined by many driving forces like globalization, urbanization, energy prices, and climate change. According to the report of the food and agriculture organization of the United Nations (FAO) 2010, the number of undernourished

people around the world in 2010 has declined but remains abnormal and unacceptable. The renewed global attention is being given to the role of agriculture and food in development policy. One of the required actions that is suggested by the Egyptian Cabinet, Information and Decision Support Center (IDSC) to solve the food problem in Egypt was to focus on the agricultural research to enhance the capability of crop plants to withstand different abiotic stresses, such as salt, drought,

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cold and Heat shock which will lead to higher yields by either increasing the crop set and/or by extending crop cultivation in the areas previously denied due to abiotic stresses.

Understanding the gene networks that represent the biological system of plants under abiotic stress and their defense mechanism makes it necessary to characterize the candidate genes that are responsible for the physiological response to the stress. Trehalose is an important building block to build up sugars that create cellular signaling and communication. It is included in the building of a number of cell wall glycolipids. Trehalose is a disaccharide sugar widely distributed in bacteria, fungi, insects, plants and invertebrate animals. In microbes and yeast, trehalose is produced from glucose where trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) function together as a large complex to synthesize trehalose. Moreover, TPS and TPP serve as sugar storage, metabolic regulator and protect living organisms against abiotic stress (Wiemken, 1990; Strom and Kaasen, 1993). Before 1997, it was thought that trehalose is present only in a few desiccation-tolerant plants. However, its role in plants was not yet fully elucidated. Under osmotic stress, trehalose was shown to accumulate at high levels in resurrection plants such as *Selaginella lepidophylla* (Wingler, 2001) and as a sugar reserve or stress protectant in *Arabidopsis thaliana* (Goddijn and Smeekens, 1998; Vogel et al., 2002; Schlupepmann et al., 2003). Interestingly, TPP and TPS genes are broadly found in the genomes of higher plants and form large gene families (Leyman et al., 2001; Schlupepmann et al., 2004). Mellor (1992) considered trehalose as a symbiotic determinant between higher plants and microorganisms. However, there is no direct evidence supporting this hypothesis so far. It was found that the precursor of trehalose, trehalose-6-phosphate, (T-6-P) is the key regulator in the glycolytic pathway (Blazquez et al., 1998). It targets the initial step of glycolysis to reduce the entrance of glucose into glycolysis. The same role of trehalose in the sugar metabolism was investigated (Vogel et al., 1998; Paul, 2001; Wingler, 2001; Eastmond and Graham, 2003). From the results of genetic and reverse genetic analysis, trehalose was found to have an essential role in carbohydrate metabolism and development of higher plants. In *Arabidopsis*, loss of *AtTPS1* function is an embryo-lethal phenotype (Eastmond et al., 2002; Schlupepmann et al., 2003; Gomez et al., 2006); and a mutation in the maize TPP gene caused abnormalities in the inflorescence architecture (Sato-Nagasawa et al., 2006). Trehalose content increased in rice as a result of the over expression of fused bacterial TPS and TPP proteins (named TPSP). The bi-functional TPSP protein enhanced the rice tolerance to abiotic stresses (Garg et al., 2002; Jang et al., 2003). Over expression of TPSP had a direct effect on the photosystem II damage under abiotic stress

(Garg et al., 2002; Jang et al., 2003). El-Bashiti et al. (2005) reported the possible role of trehalose as osmoprotectant compound in wheat species under salt and drought stress conditions. The accumulation of trehalose in wheat under abiotic stresses was found to be tissue and species specific.

Martínez-Barajas and his colleagues (2011) analyzed T6P content and *SnRK1* activities in wheat (*Triticum aestivum*) grain. The data shows a correlation between T6P and sucrose overall that belies a clear effect of developmental stage and tissue type on T6P content, consistent with tissue-specific regulation of *SnRK1* by T6P in wheat grain. Homologs of SNF1-related protein kinase1 (*SnRK1*) marker genes designated in *Arabidopsis* (Baena-González et al., 2007) was used to prove that regulation of *SnRK1* by T6P could operate *in vivo*, using Wheat Estimated Transcript Server (WhETS; Mitchell et al., 2007).

In long term, the overexpression of trehalose biosynthetic genes in wheat may seem to be promising for improvement of abiotic stress tolerant transgenic wheat.

This work aimed at the isolation, cloning and characterization of functional trehalose-6-phosphate phosphatase (TPP) gene from durum wheat under dehydration stress to investigate the trehalose 6 phosphatase (TPP) gene ability for drought tolerant in Durum wheat in order to examine the magnitude of the TPP gene response to drought stress.

MATERIALS AND METHODS

Plant materials, growth conditions and stress treatments

Durum wheat plants, (variety Sohag 3) presumably holding genes of resistance to drought were subjected to dehydration stress. Seeds of durum wheat (*Triticum turgidum*. L. var. durum wheat) were sterilized in 10% sodium hypochloride for 30 min and then rinsed with ddH₂O for 1 min. Seeds were planted in soil composed of sand and clay (1:1) for three weeks and watered daily under controlled conditions (28°C day/25°C night, 12 h photoperiod, ~500 mol m⁻² s⁻¹ photon flux density and 83% relative humidity). Drought treatment was applied as described by Ozturk et al. (2002) where, seedlings were removed from soil, washed carefully and placed on paper towels under the same growing conditions. Leaves were harvested after 2, 4, and 6 h of drought treatment, frozen in liquid nitrogen and stored at -80°C. Control seedlings were planted and grown concurrently in the same conditions without any drought regime (well-watered) then leaves were harvested at the same time and frozen in liquid nitrogen and stored at - 80°C. For the estimation of water loss, leaves were weighted at the same time intervals as that used in the dehydration experiments (zero, 2, 4 and 6 h). The ratio of the leaves weight in comparison to the control was used as indication of water loss.

Total RNA isolation

Total RNA was extracted according to Chomczynski (1993) where, 100 mg of the control and drought treated leaves (0, 2, 4 and 6 h) were ground in liquid nitrogen. 1 ml of TriPure reagent (Cat. No. 1 667 165, Roche) was added to the fine leave powder and shaken gently. The mixture was left for 5 min at room temperature before

adding 0.2 ml of chloroform. The mixture was left at room temperature for 10 min then centrifuged for 15 min at 4°C. Half ml isopropanol was added to the aqueous phase and incubated at room temperature for 10 min. The samples were centrifuged at 12,000 x g for 10 min at 4°C. The RNA pellets were re-suspended in 75% ethanol then centrifuged at 7500 x g for 5 min at 4°C. The RNA pellets were dried and re-suspended in diethylpyrocarbonate (DEPC)-treated RNase-free water and stored at -80°C.

Reverse transcription PCR (RT-PCR)-based cDNA cloning

A pair of primers, (5'-ATGGATTTGAGCAATAGCTC-3' and 5'-ACACTGAGTGCTTCTTCCAT-3') were synthesized and used to perform RT-PCR amplification using ImProm-IITM reverse transcription system (Cat. No. A3800, Promega). According to Liang and Pardee (1995), a cDNA of the *TPP* gene was generated using a RT-PCR based approach. The PCR cycle condition consists of three segments. The first one was a pre-denaturation for 4 min at 94°C. The second variable segment was consists of 40 cycles each one was 1 min at 94°C, 1.5 min at 55°C and 2 min at 72°C; the last segment was an extension for 10 min at 72°C. The amplified cDNA fragment was purified and cloned for sequencing.

Cloning of PCR product

The PCR products were cloned in pGEM-T Easy plasmid (Promega, USA) and transferred into *Escherichia coli* DH5 α . The white colonies were picked and screened for the presence of the cloned gene of interest through digestion with *EcoRI* (Sambrook et al., 1989). The pGEM®-T plasmid having *TPP* cDNA was selected by PCR using T7 and SP6 primers that amplify the 945 bp fragment having the *TPP* gene. In this reaction, 1.25 units Taq DNA polymerase, 20 pmol primers and 200 mM dNTPS were added to 15 ng of plasmid in a buffer containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris -HCl-pH 9 and 0.1% Triton® X-100. The PCR cycle condition consists of three segments. The first was a pre-denaturation for 4 min at 94°C. The second variable segment was of 40 cycles each one was 1 minutes at 94°C, 1 min at 55°C and 2 minutes at 72°C; the last segment was an extension for 7 minutes at 72°C.

DNA sequencing and Bioinformatics analysis

The *TPP* clone was sequenced according to Sanger et al. (1977) using a Big Dye Terminator Cycle sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 DNA sequencer (Applied Biosystems). A homology search was performed using BLASTX against the NCBI protein database (<http://www.ncbi.nlm.nih.gov>). Sequences of the trehalose phosphate phosphatase genes that showed similarity to the *TPP* gene were obtained from the NCBI non-redundant and dbEST data sets using BLASTX or BLASTP ver. 2.0.10 (Altschul et al., 1997). The full amino acid sequences of the proteins were aligned using CLUSTAL- X ver. 1.8 (Thompson, et al., 1997) and subjected to phylogenetic analysis. Phylogenetic trees were constructed using the neighbor-joining (NJ) method (Saitou, and Nei, 1987) with parsimony and heuristic search criteria and 1000 bootstrap replications to assess branching confidence.

Rapid amplification of cDNA PCR (RACE-PCR)

Rapid amplification of cDNA PCR was done to obtain the full length of *TPP* gene. According to Frohmann (1994), preparation of cDNA and anchor primers was conducted using Roche kit cat No.

1734792 (Clontech Lab, Inc). PCR was performed by using SP2 primer from advantage cDNA PCR kit (Clontech) and the primer 5'-CCTCCAGCACTTCGTTTACGAG-3' designed according to the gene sequence. PCR products were migrated by electrophoresis on 2% (W/V) agarose gel. The glass-milk (BIO 101) was used to recover and purify the DNA fragment, which was then ligated to pGEM®-T easy vector and finally transferred into *Escherichia coli* DH5 α (Invitrogen, cat.No.18265-017). the cloned full length gene was sequenced using ABI PRISM big dye terminator cycle sequencing ready reaction kit (PE Applied Biosystem, USA).

Expression patterns using semi- quantitative RT-PCR

Template cDNA was prepared using Super-Script II (Invitrogen) with 1 mg total RNA. 1 μ l of cDNA reaction mixture was diluted with 9 μ l DEPC treated water, then, 1 μ l of diluted mixture was used to perform Semi-quantitative RT-PCR reaction as follows: 1.0 μ l dNTPS (10 mM), 2.5 μ l MgCl₂ (25 mM), 5.0 μ l 10X buffer, 5.0 μ l Forward primer (10 pmol/ μ l), 5.0 μ l Reverse primer (10 pmol/ μ l), 1.0 μ l Template cDNA (25 ng/ μ l), 0.5 μ l *Taq* (5 U/ μ l), up to 50 μ l dd H₂O. The amplification was carried out in Hybrid PCR Express system programmed with specific primers for *TPP* and *18S* (as a control to normalize the amount of cDNA present in each sample) genes as follows: 5 min at 95°C, followed by 35 cycles at 95°C for 45 s, 55°C for 60 s, 72°C for 2 minutes, 72°C for 5 minutes. For each sample, 10 μ l of the amplification reaction was size-fractionated on a 2% (w/v) agarose gel and stained with ethidium bromide. Bands were detected on UV-transilluminator and photographed by a Gel Documentation system 2000 Bio-Rad to ensure that amplifications were in the linear range, for each template and primer pair. A Gene Ruler™ 1 kb DNA ladder was used as a standard.

Real-time PCR data analysis

Primers of *TPP* and *18S* used for semi-quantitative RT-PCR were used in real time PCR analysis. The most commonly used method for relative quantification is the 2^{- $\Delta\Delta C_t$} method. Derivation and examples of this method have been described by Livak and Schmittgen (2001). The relative difference in gene expression using the 2^{- $\Delta\Delta C_t$} method was calculated as follows:

Relative fold change in gene expression = 2^{- $\Delta\Delta C_t$} ,
Where, $\Delta\Delta C_t$ = ΔC_t treated - ΔC_t untreated and ΔC_t = (C_t target gene - C_t reference gene).

Trehalase enzyme assay

Samples weights about 100 mg from drought treated leaves at 0 hr (control), 2, 4, 6 hrs, were ground in liquid nitrogen. The powder was suspended in ice-cold suspension solution containing 0.1 M citrate (Na⁺), pH 3.7, 1 mM PMSF, 2 mM EDTA and insoluble polyvinylpyrrolidone (10 mg/g dried weight)). 2 ml of extraction buffer was added to each 1g dry weight of sample. The homogenate was filtered through two layers of cheesecloth and centrifuged at 31,500 rpm (48,000 g) for 30 min at 4°C in Sorval Combi Plus with T-880 type rotor. The supernatant was used for the enzyme assays. Adapted from Vandercammen et al. (1989). The protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Trehalase enzyme activity was measured using glucose oxidase-peroxidase kit (Bicon) according to Müller et al. (1992). The reaction mixture was composed of 10 mM trehalose, 50 mM MES (K⁺), pH 6.3 and 0.2 mg crude extract in a final volume of 1 ml.

Table 1. Leaves' weight of durum wheat under dehydration shock treatment.

Time of dehydration shock (h)	Loss in water content of leaves (%)
Control 0	0
2	15.79
4	26.36
6	34.5

The reaction was incubated at 37°C for 30 min and then started by the addition of trehalose to the reaction mixture, which was preincubated at 37°C for 10 min. 100 µl of samples were taken from the reaction mixture and immediately put in thermostat at 100°C for 3 min to stop the reaction. Precipitates were removed by centrifugation at 8700 rpm for 10 min. For the analysis, 10 µl of the supernatant was mixed with µl of glucose oxidase - peroxidase kit solution, mixed by vortex and then the mixtures were incubated at 37°C for 15 min. The absorbance of the samples was measured at 470 nm in Shimadzu UV-1201 spectrophotometer against blank solution. The increase in the absorbance against time was assumed to be equal to the amount of glucose formed. One unit of trehalase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 mmole of trehalose/ minutes at 37°C at pH 6.3.

In silico* and comparative mapping of *TPP

For *in silico* mapping, the isolated sequence was compared to rice and oat sequences using BLAST (with an e-value threshold of 1e-1000). The matches were used to identify markers from the genetic linkage map (<http://www.tigr.org>). The results obtained from this stage were used to construct a comparative map between durum and bread wheat, rice, sorghum, barley and oat to identify the tentative chromosomal location of the gene under study using comparative mapping strategy (Diab et al., 2007; Abou Ali et al., 2009).

RESULTS AND DISCUSSION

Physiological parameters and dehydration stress-specific transcript profiles

As shown in Table 1, leaves' weight was gradually decreased with dehydration time compared with the control (zero time dehydration). This weight losses indicates the decline in the water content of experimented leaves by 15.79, 26.36 and 34.5% for 2, 4, 6 h dehydration respectively. These results are in agreement with Ozturk et al. (2002) who found that the water content of barley declined by 10% within the initial 4 h, and then more rapidly by 30% (6 h) and 36% at 10 h of stress. Xue et al. (2008) reported that the change in relative leaf water content (LWC) of different genotypes indicated their different susceptibility to water scarcity. The dehydration treatment provides reliable, fast and easy way to detect genes responsible to abiotic stress response in physiological term (Talame et al., 2006).

Molecular cloning of *TPP* fragment

The RT-PCR reaction produced by *TPP1* gene fragment

with a length of \approx 1000 bp is shown in Figure 1. The amplified *TPP* cDNA fragment was ligated into the pGEM-T easy vector (3015 bp) and transformed in *Escherichia coli* competent cells. The cloned *TPP1* fragment was screened using T7 and SP6 primer. Positive colonies having the insert displayed a band about \approx 1300 bp (*TPP* fragment with a length of \approx 1000 bp linked to the region between Sp6 and T7 in native pGEM-T easy plasmid \approx 300 bp) (Figure 2).

Sequence analysis of trehalose-6-phosphate phosphatase (*TPP*) fragment

The isolated fragment was sequenced using ABI PRISM. Figure 3 shows the sequence obtained for the *TPP* fragment. Sequencing of the isolated fragment revealed that the length of Trehalose-6-phosphate phosphatase (*TPP*) fragment was 945 bp. The obtained sequence was subjected to the BLASTx analysis which proves that the sequence has different degrees of similarity with other *TPP* genes. The *TPP* fragment showed similarity to the *TPP* genes from *O. sativa*, (EU559275.1) 88%, *A. thaliana* (AY093147.1) 66% and *Z. mays* (NM_001158750.1) 66%.

Isolation, cloning and characterization of the full-length *TPP* gene

first strand of cDNA was synthesized according to Frohmann (1994) from total RNA using a gene specific cDNA primer SP2, (5'GGACGAACCTCTAAAACCATTC3'). The terminal transferase was used to add a homopolymeric A-tail to the 3' end of the cDNA. Since eukaryotic coding sequences and 5'untranslated RNA regions tend to be biased toward G/C residues, the use of a poly (A)-tail decreases the likelihood of inappropriate truncation by the Oligo dT-anchor primer. Additionally, poly(A)-tail was used due to the weaker A/T binding than G/C binding, therefore longer stretches of A residues were required before the Oligo dT-anchor primer will bind to an internal site and truncate the amplification product. As shown in Figure 4, the full length of *TPP* gene that was obtained by RACE PCR was \approx 1500 bp. PCR product was ligated into PGEM-T Easy Vector and then transformed into *E. coli*. The recombinant plasmid was digested by EcoR1 res-

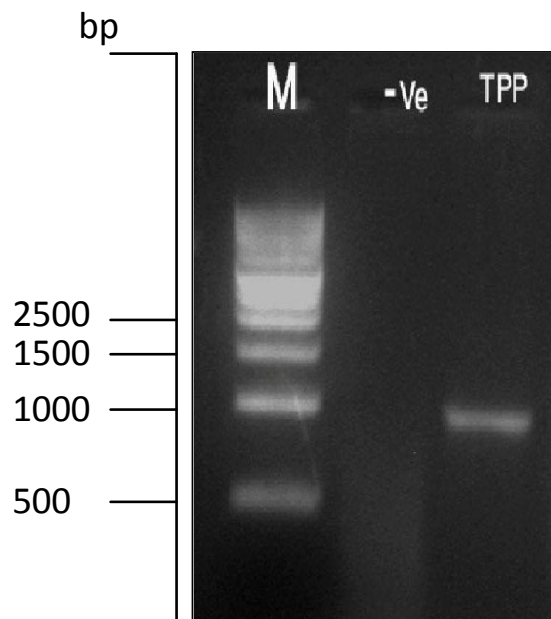


Figure 1. Agarose gel showing 1 kb marker (M), negative control (-ve), and *TPP* candidate band for *TPP* fragment.

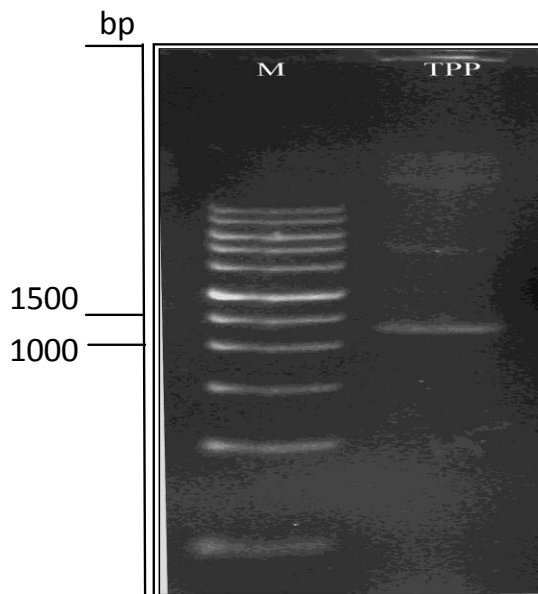


Figure 2. Agarose gel shows (M) 1 kb marker and amplified (*TPP1*) fragment using Sp6 and T7 primers.

triction enzyme to release the cloned gene. Two bands were obtained as a result of the digestion reaction of the recombinant plasmid. One was around 3000 bp representing the vector (3015 bp) and the other was around 1500 bp representing the insert (Figure 5).

The *TPP* gene(s) were isolated before in several studies. Shima et al. (2007) isolated *OsTPP1* and *OsTPP2* representing the two major trehalose-6-phosphate phosphatase genes expressed in rice, and they found that the rice genome contains nine *TPP* genes. The *OsTPP2* gene encodes a 42.6 kDa protein (382 amino acid residues). The same results were obtained by Pramanik and Imai (2005). They found nine putative *TPP* genes in the rice genome sequence. In *Arabidopsis*, 11 *TPS* and 10 *TPP* genes have been identified (Leyman et al., 2001; Eastmond and Graham, 2003). While Alexandrov et al. (2009) isolated 1883 bp trehalose-phosphate phosphatase from *Z. mays* and Ge et al. (2008) isolated 1478 bp *TPP1* gene from *O. sativa*.

The isolated full length gene was sequenced using ABI PRISM (310 Genetic Analyzer); the sequence data is shown in Figure 6. This sequence was utilized to run a homology search using blast tool provided by NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al., 1997). The results of the homology research revealed that the isolated gene displayed different degrees of similarities to other *TPP* genes. The isolated durum wheat *TPP* showed similarity with the *O. sativa*, (AB120515.1) *TPP* by 93%, *A. thaliana*, AY059840.1 by 68%, *Z. mays* (NM_001158750.1) by 76%.

According to the open reading frame of the isolated gene, the length of the protein that was expressed from

the isolated gene was of 481 amino acid residues. The deduced amino acid sequence of the isolated *TPP* gene had a molecular weight of 53546.61 Daltons, and its isoelectric point was 10.37. The amino acid analysis results of *TPP* protein revealed that it contains 71 strongly basic amino acids, 40 strongly acidic amino acids, 172 hydrophobic amino acids, and 127 polar amino acids. Amino acids sequence of the *TPP* was analyzed using BLASTP. The results show that these sequences have high homologous sequence with different *TPP* amino acids sequences. The Durum wheat *TPP* had a similarity of 93% to *O. sativa* (AB120515.1), to *Nicotiana tabacum* (BAI99253.1) by 56%, *A. thaliana* (NP_193990.1) by 56%, *Z. mays* (NP_001152222.1) by 53% and *Arabidopsis lyratanigra* (XP_002887719.1) by 42%.

Alignment of the predicted amino acid sequence of *TPP* with proteins from other species identified several conserved regions as shown in Figure 7. Pramanik and Imai (2005) reported that the alignment of the *OsTPP1* protein sequence with other *TPP* gene products from *Saccharomyces cerevisiae* (*ScTPS2*), *E. coli* (*EcOtsB*) and *Arabidopsis* (*AtTPPA* and *AtTPPB*), revealed that *TPP* sequences are moderately conserved with exception in the N-terminal region. The two distinct phosphatase boxes that are unique features of phosphatases are highly conserved (Vogel et al., 1998). Eastmond et al. (2002) investigated the *TPP* and *TPS* multigene family in plant sequences and suggested that trehalose biosynthesis is highly regulated by environmental changes in plants. Van Dijck et al. (2002) reported that, all *TPS* proteins in plants contain a conserved N-terminal extension that not found in fungal or bacterial *TPS* proteins.

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TTTGTTAAACTAAAGGAGCTTAAGTAT AACGGAAACCATGACACAGCCACCGTATCA
AATTTCT CAGAGGCATAA CAACATGACT AATAATAGTAAA CAAGCCAATATC AAT
CGACCTA AACGCAATTTTCTAA CAGGGGACGG CAACATCATT CGACCCTAGC TAT
GGATTGCC AACGCTGCAAAACAGTCGCCTGTCGGAAACTA CAGTCATCTC CTGTTT
GTTT ATTATCGCAACGTTGCAGCGAGTG CTTGGAAACTGGTCTTCCATCTCACCAAG
G AGTTCAGGAACGCT CTCCATCACT TCAGACGGGT CCCTGAGCGA GTAGAAGGCC
TCGGATTCTT TCGGCGCCTG ACGAGACCAGT ATCCCGTATC CGCAGTTCCT CTCCC
GAAGCACCTTGACTACG CGTCTTCGTC GGTGCGGTCG TCGCCGATGT AGATAGGGA
T CACTTTCTCG GACTCGCTCA GCGCCAGCGA CTGAAGCAGG AATCGACGGC CTTC
CCCTTG TCCCAGTCAAGAAGAGATCACTGGACG AACCTCTAAA ACCATTCTGTC CGT
TGGGAGATCAC TTTGAGACGG GGAAGTCTT CCAGCACTTC GTTTGACGAGC CGT
GCGACCA GCTCCCAGTCAAGAAGA CTTCTCGTCC ACGTTGCGGTAATGTACAGA CA
CGCAGAAC TTGTTGTAGCCT CAACGCTTGC GCCTTCGATT CGGCTCGTGACTTCCA
AGAG GACCTTGAA ACCTCATCGA TCATAGGCAG AAAATCGCGA GCAGTTGGAA
GAGGTTGGCT TCTTTGCCCT TTTCAGCATT GCGTTCATAA TGTGCTGAAG GATGGT
TCATTA TGTCCATCGCC ATTGTAGACTACCA GCATAGCAGA GTTCCTTCAG ATTTA
CGAAT TCAAGAACCTTATTCGGGGA CCTTCCATTG TCCGTGTCAA AAAGAAAAAA
AGTAAAAAA

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Figure 3. Nucleotide sequence of (*TPP*) fragment representing 945 bp.

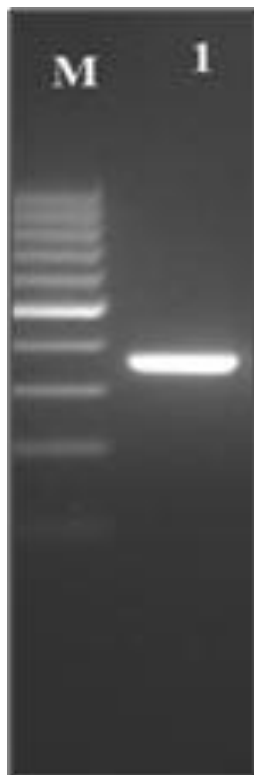


Figure 4. Agarose gel electrophoresis of gene-specific fragment (*TPP1*) gene ≈1500 bp isolated by RACE-PCR.

To determine the evolutionary relatedness of *TPP* protein to Trehalose 6 phosphate phosphatase proteins isolated from other species, the neighbor joining method

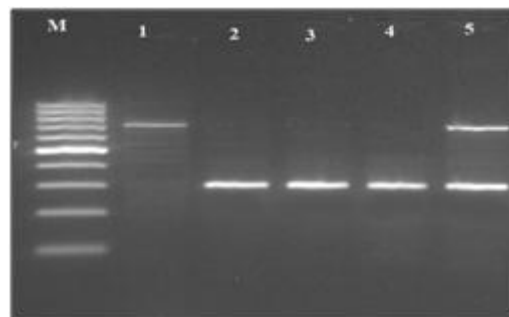


Figure 5. Agarose gel representing digestion of candidate colonies with *EcoRI* enzyme, 1kb marker (M), pGEM-T vector (1), (*TPP*) gene (2, 3, 4) and positive colony plasmid (5) DNA digestion.

(NJ) was used to generate a phylogenetic tree based on amino acid sequence homology. The tree showed that *TPP* protein forms a distinct clad on phylogenetic trees derived from various *TPP* sequences (Figure 8). Bootstrap analysis placed the durum wheat (*Triticum durum*) sequence close to *O. sativa* with a high degree of confidence, demonstrating that the two species descent from common ancestor.

Protein sequence analysis homology modeling

The results of BLAST search against PDB program exhibited a high level of sequence similarity to the crystallized structure for modeling *TPP* protein (Figure 9). This protein structure is very important to study the mode of action of disaccharides, like trehalose that appear to be one of the most effective stabilizers for dried enzymes

(A)
 ATGGATTTGA GCAATAGCTC ACCTGTCATC ACCGATCCGG TGTCGATCAG CCA
 GCAGCTGTTGCGCGCCC TGCCTTCAA TCTGATGCAG ATTTTCAGTCA TGC GCGG
 TGG C TACTCCAGCTCTCGCATGG ACGTTGGTGT CAGTAGGCTC ATAATCGAGG
 AAGACCTTGTCAATGGACTGCTTGATGCGA TGAAATCCTG CTCACCTCGC AGGA
 GGCTGA GTGTAGCAAT TGGCGAGGACAATTCATCTG AAGAAGATGACGCTGCTT
 AC AGCGCTTGGG TGGCAAATG TCCTTCTGCATTGGCTTCT TCAAGCGAAT TG
 AAGCGAGT GCACAAGGGA AGGAGATTGC TGTGTTTCTAGACTATGACG GCACA
 CTGTC GCGTATTGTG GATGATCCTG ACAAAGCAGT GATGTCTCCCCTGATGAGT
 G CTGCTGTGAG AGATGTTGCG AAGTACTTCC CCACTGCAAT TGTCAGCGGA A
 GGTCCCGCA ATAAGGTGTTTGAATTTGTTAAACTAAAGGAGCTTAAGTAT AACG
 GAAACCATGACACAGCCACCGTATCAATTTCT CAGAGGCATAA CAACATGACT A
 ATAATAGTAAA CAAGCCAATATC AATCGACCTA AACGCAATTTTCTAA CAGGG
 GACGG CAACATCATT CGACCCTAGC TATGGATTGCC AACGCTGCAAAACAGTC
 GCCTGTGCGAAACTA CAGTCATCTC CTGTTTGTTC ATTATCGCAACGTTGCAGCG
 AGTG CTTGGAAACTGGTCTTCCATCTCACCAAGG AGTTCAGGAACGCT CTCCAT
 CACT TCAGACGGGT CCCTGAGCGA GTAGAAGGCCTCGGATTCTCT TCGGCGCCTG
 ACGAGACCAGT ATCCCGTATC CGCAGTTCTCTCCCCGAAGCACCTTGACTACG C
 GTCTTCGTC GGTGCGGTGCG TCGCCGATGT AGATAGGGAT CACTTTCTCG GACTC
 GCTCA GCGCCAGCGA CTGAAGCAGG AATCGACGGC CTCCCCTTG TCCCAGTC
 AAGAAGAGACTACTGGACG AACCTCTAAA ACCATTCGTC CGTTGGGATCAC
 TTTGAGACGG GGAAGTCCT CCAGCACTTC GTTTGACGAGC CGTGCGACCA GC
 TCCCAGTCAAGAAGA CTTCTCGTCC ACGTTGCGGTAATGTACAGA CACGCAGAA
 C TTGTTGTAGCCT CAACGCTTGC GCCTTCGATT CGGCTCGTGACTTCCAAGAG G
 ACCTTGAA ACCTCATCGA TCATAGGCAG AAAATCGCGA GCAGTTGGAA GAG
 GTTGGCT TCTTTGCCCT TTTTCCAGCATT GCGTTCATAA TGTGCTGAAG GATGGTT
 CATT TGTCCATCGC ATTGTAGACTACCA GCATAGCAGA GTTCCTTCAG ATTT
 ACGAAT TCAAGAACCCTATTCGGGGA CCTTCCATTG TCCGTGTCAA AAAGAAAA
 AA AGTAAAAAA

(B)
 MDLSNSSPVITDPVSIQQLLRALPSNLMQISVMRGGYSSSRMDVGVSRLLIEEDLVNG
 LLDAMKSCSPRRRLSVAIGEDNSSEEDDAA YSAWMAKCPALASFKRIEASAQGKEI
 AVFLDYDGTLSRIVDDPKAVMSVMSAAVRDVAKYFPTAIVSGRSRNKVFVFKLKE
 LKYNGNHDATVSIQRHNNMTNNSKQANINRPKRNFLTGDGNIIRPLWIANAAKQS
 PVGNYSLLFVHYRNVAAAWKL VFHLTKEFRNALHHFRVPERVEGLGFLRRLTRP
 VSRIRSSPEAPLRVFGAVVADVDRDHFLGLAQRQLKQESTAFPLSQSRRDHWTN
 LNHSSVGRSLDGGSPALRLTSRATSSQSRLLVHVAVMYRHAELVVASTLAPSIRLV
 TSKRTLETSSIIGRKSRAVGRGWLLCPFHCVHNVLKDGS LCP SPLTTSIAEFLQIYEFK
 NLIRGPSIVRVKKKSKK.

Figure 6. (A) Nucleotide sequence of (*TPP1*) gene representing 1458 bp as obtained from the ABI PRISM 310 DNA sequencer. (B) The amino acid sequence (481 aa).

and cell membranes *in vitro* and *in vivo*. However, the interaction of trehalose with biological membranes has been studied more than its interactions with other proteins.

Carpenter (1993) has reported that trehalose might interact with dry protein by hydrogen bonding to the polar amino acid residues in the protein. On the other hand, the interaction between trehalose and biological membranes indicates that trehalose can replace H₂O molecules around the polar head groups of the phospholipid in the dry state (Gaber et al., 1986). This hypothesis has been studied by Potts (1994) where trehalose binds to dry phospholipid vesicles. During desiccation, the interaction of trehalose with the biological membrane decreases the melting temperature (T_m) of the membrane to keep its

liquid crystalline phase (Crowe et al., 1993). This molding could be used to predict the interaction between the trehalose and other protein candidates in the biological membranes for more understanding of the trehalose mode of action for protecting plants against abiotic stress conditions.

Expression analysis

The results obtained in this work indicate that the expression of the *TPP* gene was up-regulated under dehydration stress compared to control (Figure 10). The highest expression level of *TPP* gene under dehydration stress was at 4 h. This up-regulation is important for the synthesis and accumulation of trehalose where, trehalose

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OsTPP 1 ---MDLSNS--SPVITDPVAISQQLGGLPSNLMQFSVMPGGYSSSGMN--VGVSRLK----IEEVLVNGLLDAMKSSS
TPPnew 1 ---MDLSNS--SPVITDPVAISQQLGGLPSNLMQFSVMPGGYSSSGMN--VGVSRLK---PKLDDVRSNGWLDAMISSS
ArTPP 1 ---MDLNINKTTPVLSDPPTTPVSKTRL-----GSSFPSSGRFM--MNSRKKI---GLDDVRSNTWLDAMKSSS
NTTPP 1 ---MDLKSNTSPVITDPVAISQQLGGLPSNLMQFSVMPGGYSSSGMN--VGVSRLK---PKLDDVRSNGWLDAMISSS
ZMTPP 1 ---MDLKTGLNSPVIADHLPTLALPAAVMTFT-----TPTSPFSPGLC--LNTTKKIPLNVLVWAPKITSLSIDSMRDS
ArLTPP 1 MTNQNVIVSDRKFILGLKTIIVSVTNSPLFNSFP---TYFNFPRRKLKLLLEVADKNNN-----

OsTPP 69 P-RRRLNVAFGEDNSSEEDPAVSAWMAKCPALASFKQIVASAQGRKIAVFLDYDGTLSPIVDDPKAVMS PVMRAAVR
TPPnew 71 PPRKRLVKDFNIEIAP-EDDFSORAWMLKYPSAITSFAHIAAQAKNKIAVFLDYDGTLSPIVDDPDRAIMSDAMRAAVK
ArTPP 61 PTHSKKNKDSNAELTANESDLAWRIWMLKYPSALS SFEQITNYAKGRRIALFLDYDGTLSPIVDDPDRAFMS GAMRAIVR
NTTPP 72 PTRKRQIKDVICDAQS-DLDLQVCNWTVNYPSALISFEAISDLAGSKRLALFLDYDGTLSPIVDNPNALMSDEMRAAVR
ZMTPP 70 PTRLRSS--SHDSVSDIDDKTS--WIVRFPALNMFDEIWNAAKGRQIVMFLDYDGTLSPIVDDPKAFITHEMRDVKW
ArLTPP -----

OsTPP 148 NVAKYFPTAIVSGRSRNKVFEFVKLELYYAGSHGMDIMAFSANHEHSAE-----KSKQANLFPQPAHDFLPMIDEV
TPPnew 150 DVAKYFPTAIIISGRSRDKVYQLVGLTELYYAGSHGMDIMTPVNPNGSPEDPNCIKTTDQQGEEVNLFPQPAKEFIPVIEEV
ArTPP 141 NVAKYFPTAIIISGRSRDKVYDFVGLAELYYAGSHGMDIMGPVR--SVSDDYSCIKFTDKQKGEVNLFPQPAEFLPMIDEV
NTTPP 151 HAASLFPTAIIISGRSRDKVDFVKNLELYYAGSHGMDIMGPVRKTTD SNGVECIIRSTDVHGKEVNLFPQPAEFLPMIDEV
ZMTPP 145 DVASNFPTAIVTGRSIDKVRSFVKVNEIYYAGSHGMDIEGPTNENNYGQS-----NLRVLFQPAEFLPMIEKV
ArLTPP -----

OsTPP 219 TKSLLQVVSGLIEGATVENNKFCVSVHYRNVAEKDWKLVARLVNEVLEAFPRLKVTNGRMVLEVRPVIENWKGKAVEFLE
TPPnew 230 YNNLVELTKIKGAKVENHKFCISVHYRNVDEKDWPLVAQRVHDHLKRYPRLRITHGRKVLEVRPVLNWDKGKAVEFLE
ArTPP 219 FRSLVELTKDITGAKVENNKFCVSVHYRNVDEKSWSAIGESWDELKHYPRLRLTHGRKVLEVRPVIDWNKGKAVEFLE
NTTPP 231 YEKLGESVKDIDGARMEDNKFCVSVHYRNVAEEDYKKVFRHTAVLEGYPCRLRLTHGRKVLEIRPTIKWDKGOALNFLK
ZMTPP 214 IKILEEETKWIISGAMVENNKFCISVHFRRVDEKRWITALAEVWKSVLIDYPELKLTKGRKV
ArLTPP -----

OsTPP 299 SLGLSNNDEFLPIFI GDDKTD EDAFK VLREGNRGFGILVSSVPKESNAFYS LRDPSEWKKFLKTLVWKGKMESSKTSF
TPPnew 310 SLGLKNCDDVLPYIYV GDDRTD EDAFK VLREGNKGYGILVSSAPKESSAFYS LRDPSEWMEFLKCLVSWKKS SGF SN--
ArTPP 299 SLGLSESEDVLPYIYV GDDRTD EDAFK VLKASNRGFGILVSSVPKESDAFYS LRDPAEWMEFLRMLAAWKEQST-----
NTTPP 311 SLGYEDSEDVVPYIYV GDDRTD EDAFK VLREERGQFGILVSKVPKDTNAFYS LRDPSPQWKKFLERLVEWKKRKTVGE E--
ZMTPP -----
ArLTPP -----
    
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Figure 7. Alignment of the predicted amino acid sequence of *TPP* and those from other plants including *Arabidopsis lyrata* (*ArLTPP*), *Zea mays* (*ZmTPP*), *Arabidopsis thaliana* (*ArTPP*), *Nicotiana tabacum* (*NtTPP*), *Oryza sativa* (*OsTPP*).

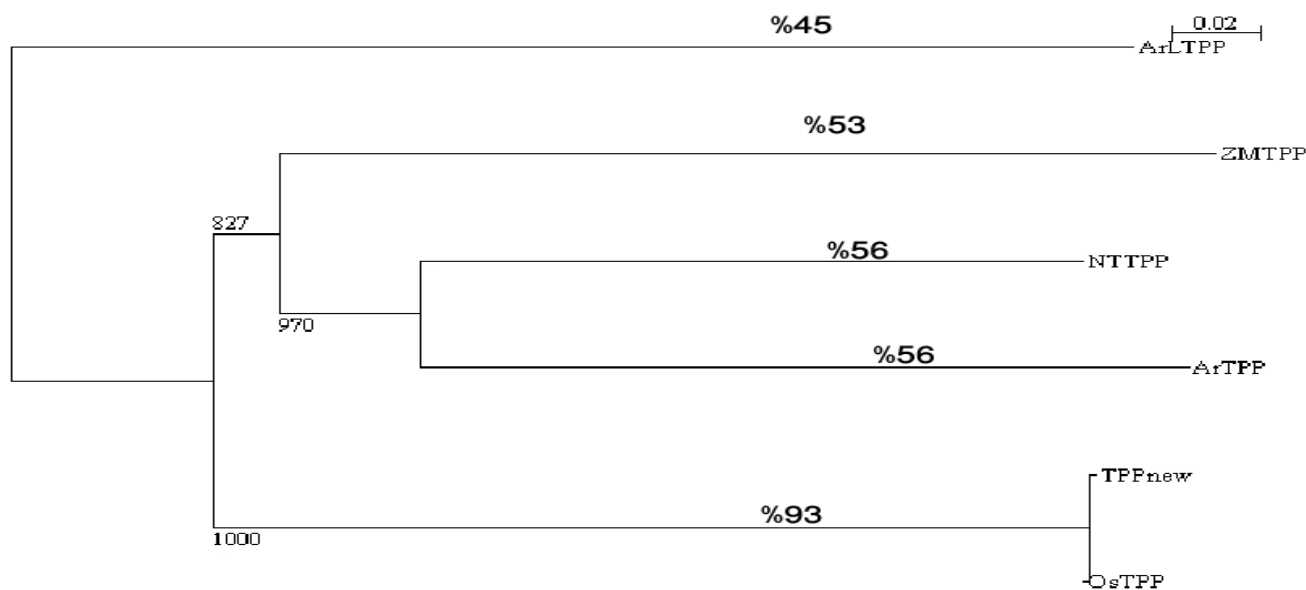


Figure 8. Phylogenetic tree based on sequences of *TPP1* with other *TPP*s isolated from other plants. The phylogenetic tree was constructed by the neighbor-joining including *Arabidopsis lyrata* (*ArLTPP*), *Zea mays* (*ZmTPP*), *Arabidopsis thaliana* (*ArTPP*), *Nicotiana tabacum* (*NtTPP*), *Oryza sativa* (*OsTPP*).

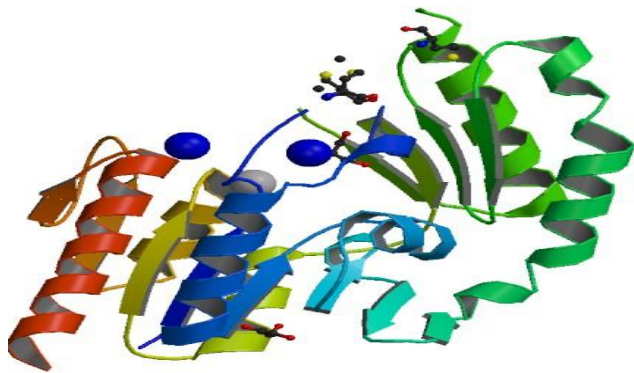


Figure 9. 3D Model of *TPP* protein structure.

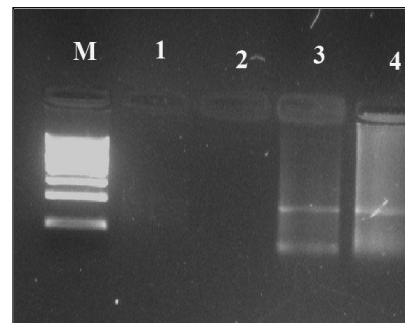


Figure 10. Agarose gel represents the *TPP* expression patterns of leaves under dehydration stress treatment after 0 (1), 2 (2), 4 h (3), 18S gene (4). M is 1Kb ladder DNA marker.

is accumulated in large quantities under abiotic stresses (Elbein et al., 2003 and Wolf et al., 2003).

Reserve transcription combined with the polymerase chain reaction (RT-PCR) has proven to be a powerful method to quantify gene expression according to Murphy et al. (1990). Real-time PCR technology has been adapted to perform quantitative RT-PCR (Heid et al., 1996). The results of the real time PCR of the *TPP* gene showed that the expression level of *TPP* was slightly increased (up-regulated) after 4 h of dehydration treatment in leaves of Durum wheat compared with the control (0 h) and the relative fold change calculated by $\Delta\Delta CT$ method, respectively which in agreement with the semi-quantitative PCR results (Figures 11 and 12). The plant's response to dehydration is accompanied by the activation of a group of genes, which are responsible for regulatory proteins that further regulate the transduction of the stress signal and modulate gene expression (Shinozaki and Yamaguchi, 2006).

Higo et al. (2006) studied the expression of trehalose gene synthesis (*mts* and *mth*, encoding maltotriooligosyl trehalose synthase and hydrolase) and trehalose hydrolysis (*treH*) in *Anabaena* sp. The genes (*mts* and *mth*) were up-regulated markedly upon dehydration. Gene disruption of *mth* resulted in a decrease in the trehalose level and in tolerance during dehydration stress. In contrast, gene disruption of *treH* resulted in an increase in both the amount of trehalose and tolerance. Trehalose did not stabilize proteins and membranes directly during dehydration; the expression of the two genes, one of which encodes a cofactor of a chaperone DnaK, correlated with trehalose content, a chaperone system induced by trehalose is important for the dehydration tolerance of *Anabaena* sp.

Cumino et al. (2002) found that many other genes, including *spsA*, encoding sucrose-6-phosphate synthase were up-regulated constantly during dehydration stress. Many genes related to photosynthesis and ribosomal protein was down-regulated in the early dehydration phase, whereas genes for nitrogen fixation and photosynthesis I was down-regulated in the late dehydration phase.

Determination of trehalase activity under dehydration stress

As shown in Table 2, the activity of trehalase enzyme under dehydration stress was decreased from 1.004 to 0.781 after 2 h and to 0.427 after 4 h. The activity was then elevated after 6 h of dehydration treatment compared to the control (Figure 13). The elevation of trehalase activity after 6 h might be due to internal regulation mechanism in the system biology of the plant to prevent the uncontrolled increase of the trehalose which is important to prevent detrimental effects of trehalose accumulation on the regulation of carbon metabolism (Brodmann, 2002).

These results are in agreement with Brodmann (2002) who showed that trehalase activity normally keeps cellular trehalose concentrations low in order to prevent detrimental effects of trehalose accumulation on the regulation of carbon metabolism. The role of trehalase may be of particular importance in interactions of plants with trehalose-producing microorganisms. In support of this hypothesis, expression of the Arabidopsis trehalase gene and trehalase activity were found to be strongly induced by infection of Arabidopsis plants with the trehalose-producing pathogen *Plasmodiophora brassicae*. Penna (2003) found that trehalose was thought to protect biomolecules from environmental stress, as suggested by its reversible water-absorption capacity to protect biological molecules from desiccation-induced damage. The low levels of trehalose in transgenic plants can be explained by specific trehalase activity, which degrades trehalose; hence, it might be possible to increase trehalose accumulation by down regulating trehalase activity. El-Bashiti et al. (2005) found that trehalase activity in different wheat cultivar was increased under control conditions in both root and shoot of Bolal cultivar compared with salt and drought stress treatments. However, under drought conditions, there was no significant change in trehalase activity of shoot tissues. Trehalase is ubiquitous in higher plants and

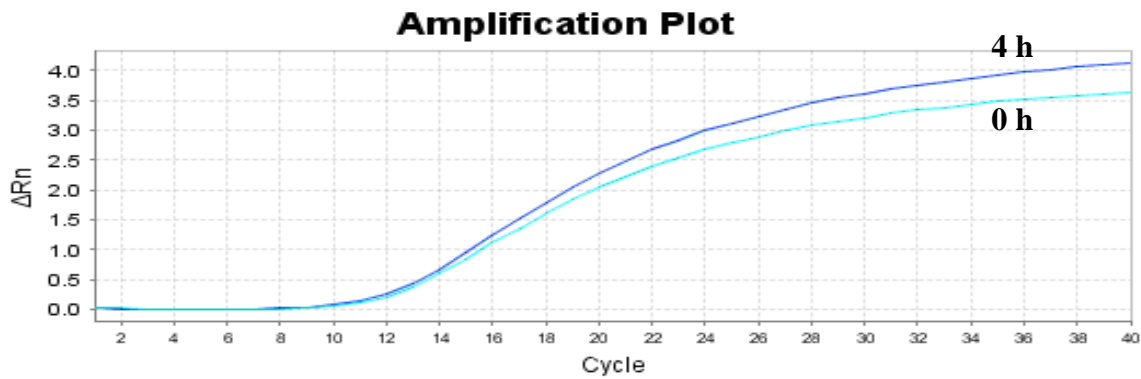


Figure 11. Variation of fluorescence amplification plot of *TPP1* gene for the dehydration shock treatments (0 and 4 h)

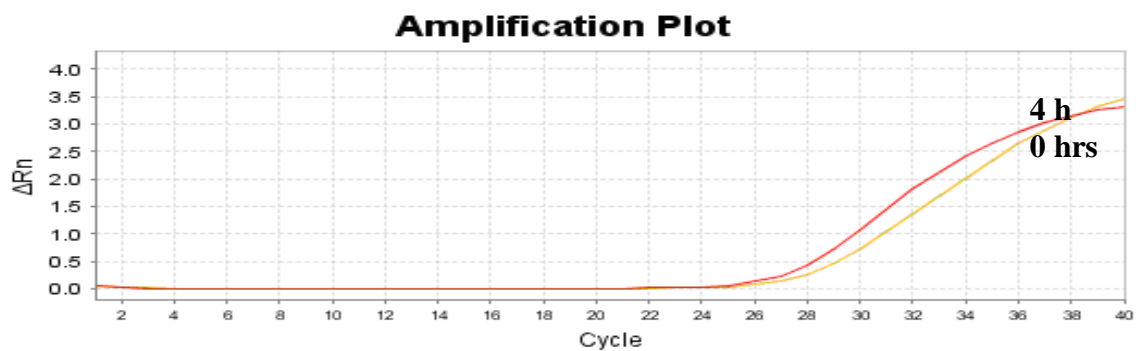


Figure 12. Normalization of fluorescence amplification plot of 18S gene (reference gene) for the three dehydration shock treatments (0, 4 h).

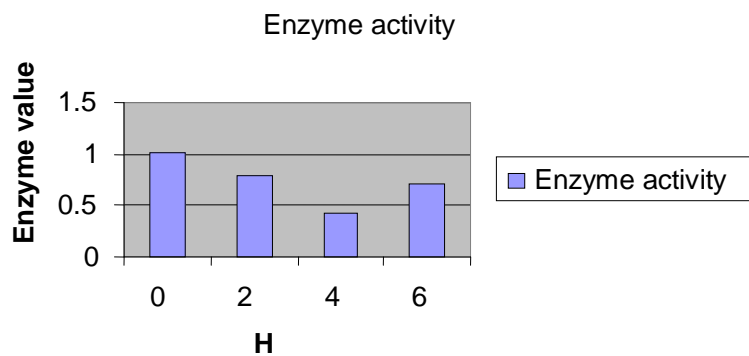


Figure 13. The trehalase activity after dehydration shock treatments.

Table 2. Trehalase activity under dehydration shock treatments.

Hour	Enzyme activity
0	1.004
2	0.7813
4	0.4273
6	0.7170

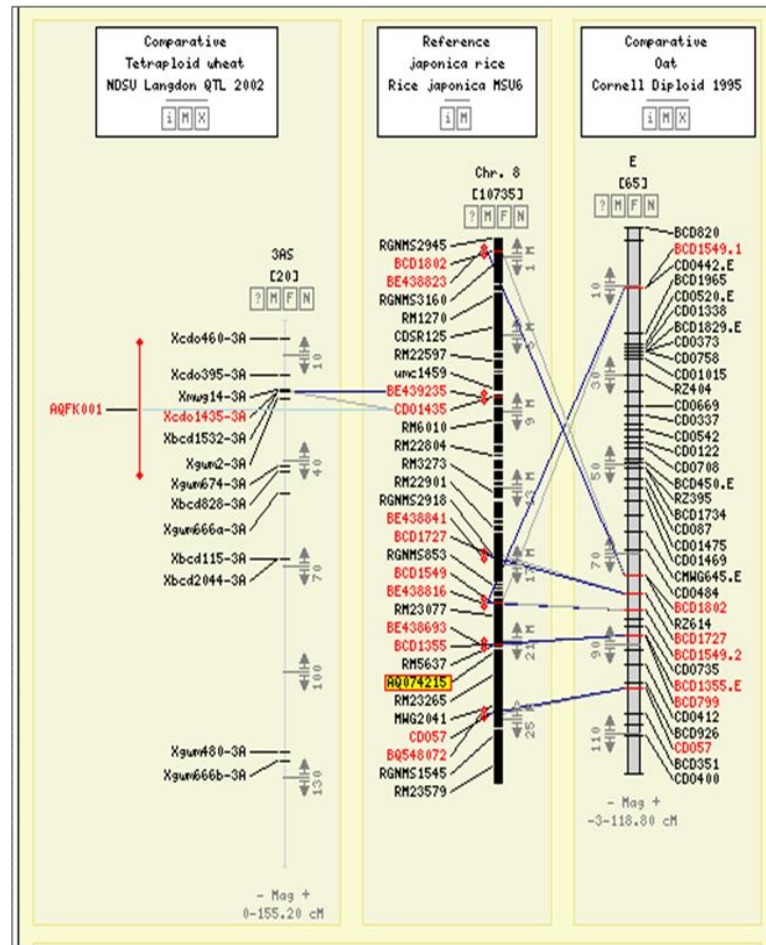


Figure 14. Comparative map showing the locus AQ074215 on Rice chromosome 8 that is closely linked to other locus on Oat and Tetraploid wheat.

single-copy trehalase genes have been identified and functionally characterized from soybean (*Glycine max*) and Arabidopsis (Aeschbacher et al., 1999; Müller et al., 2001). It is likely that trehalase is the sole route of trehalose breakdown in plants (Müller et al., 2001). Kato et al. (2004) concluded that although *TPS* catalyses the transfer of glucose from UDP-glucose to glucose 6-phosphate to produce trehalose 6-phosphate and *UDP*, and *TPP* catalyses the dephosphorylation of trehalose 6-phosphate to trehalose. The low level of accumulation of trehalose may be attributed to the unique gene structure for trehalose metabolism. They investigated the expression of genes for trehalose synthesis, *mth* (maltooligosyl trehalose hydrolase) and *mts* (maltooligosyl trehalose synthase), as well as that for trehalose degradation, *treH* (trehalase), exhibited marked increase upon dehydration. So trehalose did not accumulate so much.

***In silico* and comparative mapping**

Comparative maps can be used to study genome evolu-

tion; how the genome has been rearranged through time, and to make inferences about gene organization (Liang et al., 2008). *In-silico* mapping indicated the matching of the *TPP* gene sequence with the sequence of rice *TPP* on chromosome 8 linked to the locus (AQ074215).

Comparative mapping showed that the rice AQ074215 locus on chromosome 8 was also mapped on barley chromosome 5H. The marker (IwgsC) on bread wheat chromosome 3B was found to be closely linked to the rice locus (AQ074215) on chromosome 8. The results obtained from the comparative mapping showed that the isolated *TPP* gene is localized on rice chromosome 8, durum wheat chromosome 20, bread wheat chromosome 3B, oat linkage group E, sorghum chromosome 4 and barley 5H (Figures 14 to 16). This work utilizes a comparative analysis of durum and bread wheat, barley, oat, sorghum and rice based on linkage maps and consensus markers across the genome with the goal of linking the complex wheat genome to simpler diploid species such as barley and rice that serve as references. However, more detailed comparisons are needed to veri-

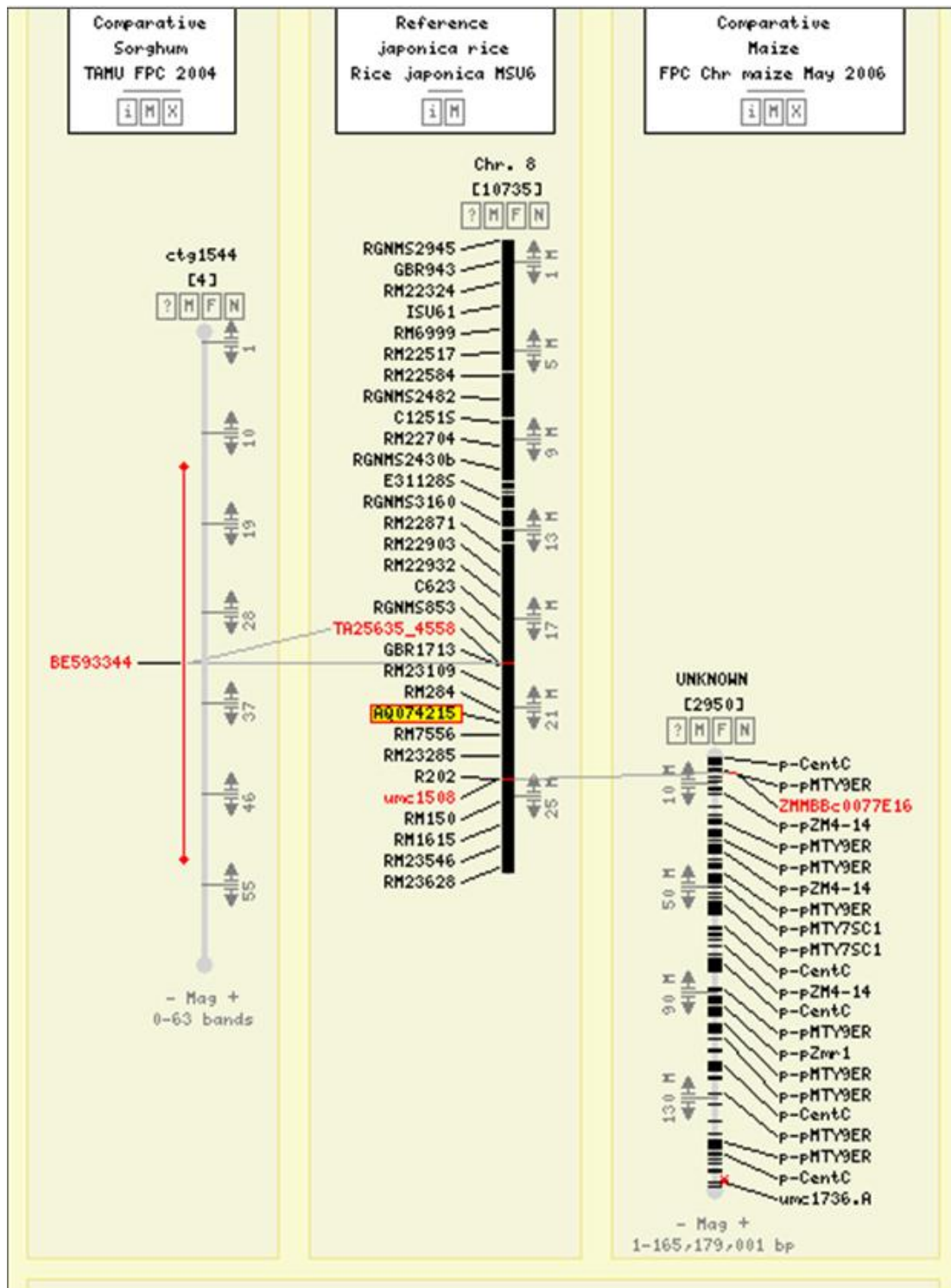


Figure 15. Comparative map showing the locus AQ074215 on rice chromosome 8 and maize and sorghum.

fy conserved regions associated the *TPP* gene.

In this study, the *TPP* gene was isolated, characterized and cloned to be used in the strategic improvement of crops for abiotic stress tolerance through genetic

transformation and the *in silico* comparative mapping of this gene would open the gate for the use of the *TPP* gene for marker assisted selection in breeding programs for abiotic stress tolerant crops.

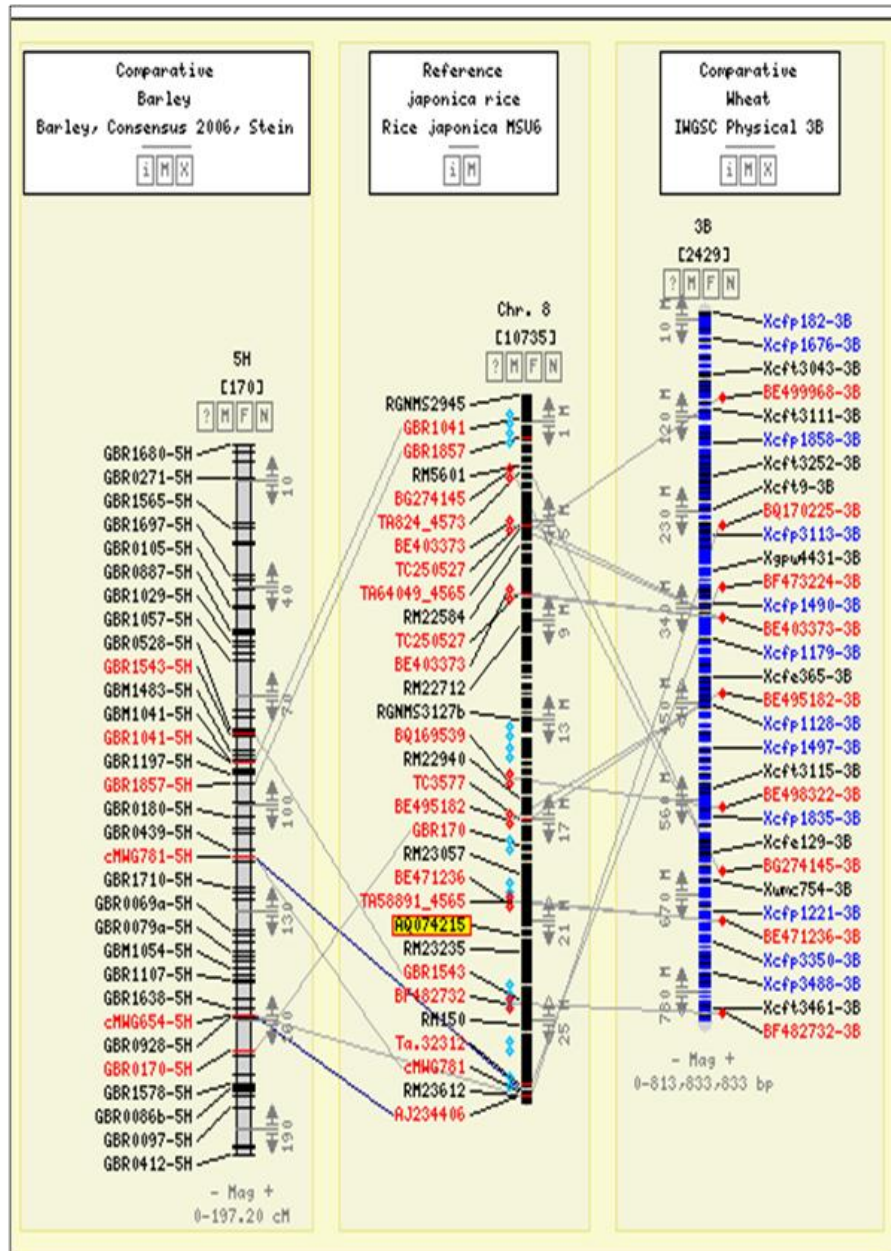


Figure 16. Comparative map showing the locus AQ074215 on Rice chromosome 8. and its linkage to barley and bread wheat.

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